4500
FS-SO-2203-1.42
(Problem 2 RWUD-1978)

October 1978
STUDY PLAN SUMMARY
J. Robert Bridges and James E. Marler
Alexandria, LA

A QUANTITATIVE STUDY OF THE MICROFLORA ASSOCIATED WITH THE SOUTHERN PINE BEETLE

(Cooperative Agreement 19-288)

Although previous studies have shown that many species of microorganisms can be isolated from the southern pine beetle (SPB), these studies did not provide information on the size of the microbial population associated with the SPB nor did they identify which of the microorganisms occur in the greatest numbers. This study will augment previous studies by quantitating the number of microorganisms present in the gastrointestinal tract of SPB adults and larvae and in bark tissue surrounding larval galleries. Microorganisms present in the greatest number will be isolated and identified. This information will be important in the study of the succession of microorganisms in SPB galleries and will be useful for determining which of the microorganisms are important to beetle development.

SO-4000-5 (REV. 9/75)

BIOMETRICS REVIEW ROUTING

ROUTING

~~1. AD-W~~
~~2. Biometrics~~
3. ~~RWU 2203~~ JRB

RESEARCH WORK UNIT

☒ STUDY PLAN ☐ MANUSCRIPT ☐ RESPONSE TO BIOMETRICS REVIEW ☐ _____ (OTHER)

FS-SO-2203-1.42 (STUDY NUMBER)

A quantitative study of the microflora associated with the Southern Pine Beetle (TITLE)

Robert Bridges / James E Marler (AUTHOR SIGNATURE) Bridges Marler (DATE)

REMARKS: For biometrician review

SUBMITTED: Peter L. Lorio, Jr. (PROJECT LEADER SIGNATURE) Peter L. Lorio, Jr. (DATE) 9/27/78

AD

☒ FORWARDED FOR BIOMETRICS REVIEW ☐ BIOMETRICS REVIEW WAIVED

D W Harrington (ASSISTANT DIRECTOR SIGNATURE) 9/29/78 (DATE)

BIOMETRICS SERVICES

☐ NO COMMENTS ☐ COMMENTS BELOW ☐ COMMENTS ATTACHED

☒ COMMENTS MADE BY PHONE ☒ COMMENTS WRITTEN ON REVIEW DRAFT

IN 10-2-78

T. R. Dell (BIOMETRICIAN SIGNATURE) (DATE)

OUT 10-4-78

COMMENTS:

INSTRUCTIONS

1. Project Leader must submit this form with each study plan. Also use this form when manuscripts are submitted for review.
2. Insert desired "Routing" in space at top of form.
3. Be sure to insert proper identification in the "Research Work Unit" block.
4. Completed copies of this form will be filed in the study file in the Assistant Director's and Project Leader's files.

ORIGINAL – RESEARCH WORK UNIT FILE

U.S. DEPARTMENT OF AGRICULTURE **SPEED-MEMO**

PART NUMBER	DATE
3	9/22/78

TO

Dr. James E. Marler
LSUA
Alexandria, LA 71301

FROM

RWU 2203
2200 Shreveport Highway
Pineville, LA 71360

SUBJECT

1630 Written Information
Study Plan "A quantitative study of the microflora associated with the southern pine beetle" (19-288)

MESSAGE

Hate to bother you again but here is another form needing your signature before we can submit the study mentioned above. I have marked the signature block for your convenience.

Biom. rev.

SIGNATURE

Bette Mallard

REPLY

SIGNATURE

DATE

(DESTROY THIS PART 3 UPON RECEIPT OF REPLY)

FORM AD-311(REV. 5-68)

UNITED STATES DEPARTMENT OF AGRICULTURE
FOREST SERVICE

SO-AL

REPLY TO: 1630 Written Information

October 17, 1978

SUBJECT: Study Plan FS-SO-2203-1.42
Bridges and Marler

TO: T. A. Harrington
AD-W

Enclosed is the above captioned study plan and blue line summary; "A Quantitative Study of the Microflora Associated with the Southern Pine Beetle".

Pete

PETER L. LORIO, JR.
Project Leader
Forest Insect Research

Encl.

PLLORIO/cm

U.S. DEPARTMENT OF AGRICULTURE **SPEED-MEMO**

PART NUMBER	DATE
2	9/18/78

FROM

Dr. James E. Marler
LSUA
Alexandria, LA 71301

TO

RWU 2203
2500 Shreveport Highway
Pineville, LA 71360

SUBJECT

1630 Written Information Study Plan "A quantitative study of the microflora associated with the southern pine beetle" (19-288)

MESSAGE

Enclosed is copy of the above mentioned study plan. Also an approval sheet for your signature is enclosed. For your convenience in returning it, an envelope, self addressed is enclosed. Once it is approved and sent to New Orleans, I will send you a final copy.

SIGNATURE Bettie Mallard

REPLY

Thanks for the Study Plan,
Now remind Bob to send me all the yeast reprints he can copy so we can "tool" up out here.

SIGNATURE

DATE

FORM AD-311(REV. 5-68)

Bridges review
9/21/78

4500
FS-SO-2203-1.42
(Problem 2 RWUD-1978)

STUDY PLAN

A QUANTITATIVE STUDY OF THE MICROFLORA ASSOCIATED WITH THE SOUTHERN PINE BEETLE

by

J. Robert Bridges and James E. Marler
(Cooperative Agreement No. 19-288 with LSUA)

INTRODUCTION

Although many types of microorganisms are associated with the southern pine beetle (SPB), the importance of most of them is not known. Three species of fungi are known to have a significant effect on beetle development. Two of these fungi are carried in the SPB female mycangium, thus ensuring a constant association with the beetle (Barras and Perry 1972; Barras, 1975a). In the absence of these mycangial fungi, SPB progeny development is delayed and progeny production is decreased by more than 50% (Barras 1975b). Thus, these fungi are mutualistic symbionts and probably play a role in beetle nutrition and possibly pheromone production (Brand *et al.*, 1976). Another fungus that affects beetle development is the blue-stain fungus, *Ceratocystis minor* which is consistently found in association with the SPB (Nelson and Beal 1929, Rumbold 1931, Barras 1970). This fungi is responsible for the death of the tree and apparently aids beetle development by speeding the desiccation of the tree (Bramble and Holst 1935, Nelson and Beal 1929, Nelson 1934).

The importance of the other microorganisms found with the SPB is not known. Previous studies have identified several species of bacteria from the gastrointestinal tracts of adult SPB and from their galleries (Barras and Marler 1974, Marler and Barras 1973, Oberle 1966, Moore 1972a and b). Bacteria representing 12 genera have been isolated (Marler and Barras 1978). Some of these are potential pathogens (Moore 1972b) and some are capable of fixing nitrogen. Other species could be fortuitious contaminants which are ubiquitous in the forest environment while others might have a significant effect on beetle development.

Yeasts are also associated with the SPB (Callahan and Shifrine 1960, Shifrine and Phaff 1956), but these fungi have received relatively little study. It has been suggested that yeasts could be important in pheromone production (Brand *et al.*, 1977), but they might also play a role in beetle nutrition by producing vitamins, for example.

Qualitative studies to date have identified the species of microorganisms but have provided no information on the number of these microorganisms present at any given time. Quantitative information of the microbial population would be useful for determining which of the organisms, if any, are important to beetle development. A quantitative estimate of microbial population would also be important in the study of the succession of microorganisms in the SPB galleries.

The purpose of this study is to estimate the number of bacteria, yeasts, and fungi* present with larvae and adults of SPB and in the phloem surrounding SPB larval galleries. Microorganisms will be identified to determine which are present in the greatest numbers. Isolations will be made in galleries of larvae of various ages to determine whether there is a succession of microorganisms.

MATERIALS AND METHODS

Beetles emerging from naturally infested loblolly pine trees will be collected using a collection box similar to that described by Brown (1972). These emergent beetles will be used to inoculate freshly cut loblolly pine bolts in the laboratory. The bolts will be kept in the laboratory for various lengths of time and sampled when the bolts contain one of the following stages:

1. Eggs
2. Young larvae in larval mines before larvae have begun to enlarge their larval chamber
3. Actively feeding larvae in the larval chambers
4. Mature outer bark larvae

---

*Fungi refers to hyphal fungi as distinguished from the yeasts.

From each bolt frass from the egg niche, phloem from three sampling points (see Figure) and eggs or larvae will be collected. In bolts containing eggs and small larvae, sampling points B and C (respectively) will be in advance of gallery construction. Three galleries of each stage will be sampled. In each case the larval galleries selected for culturing will be approximately the same distance from the adult entrance hole.

Frass, phloem, and larvae will be aseptically removed and homogenized in a tissue homogenizer. Larvae will be surface sterilized (Barras 1970) prior to homogenization in order to eliminate all but the gut microorganisms. Serial 10-fold dilutions of the homogenate will be made, and 0.1 ml of each dilution will be spread in duplicate on plates of isolation media. Isolation media will include both bacteriological and fungal media. The number of cultivable bacteria, yeasts, and fungal propagules per unit of wet weight of phloem or frass will be calculated from the average number of colonies present on the primary isolation plates. Isolated colonies will be picked and obtained in pure culture by three successive passages on streak plates. Five colonies each of bacteria, yeasts, and fungi will be picked from the primary dilution plate of greatest dilution and kept for identification. Thus, the isolates to be identified will be those that are present in the greatest numbers. Isolates of the microorganisms will be identified using standard microbiological techniques (Skerman 1969, Norris and Robbins 1969, Buchanan and Gibbons 1974).

The microflora of the guts of adult SPB will similarly be studied. Guts from 5 surface sterilized emergent SPB will be pooled, homogenized, and plated as described above. Both males and females will be studied, and the procedure will be replicated 3 times.

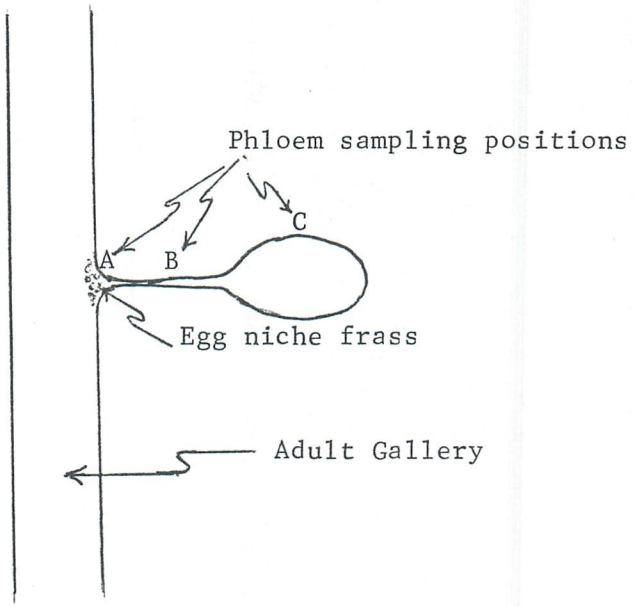

Figure. Diagram of SPB larval gallery showing sampling positions.

STATISTICAL ANALYSIS OF THE DATA

Data to be collected will include the number of cultivable bacteria (or yeasts or fungi) from each sample location. The mean number of organisms will be based on 3 replications.

	Sample positions			
Stage of bolt	Frass	A	B	C
Egg				
Larval mine				
Larval chamber				
Outer bark larvae				

These data will be analyzed using an analysis of variance as summarized in the following:

Source of variation	df	Source of variation	df
Position (rows)	3	Stage (columns)	3
Error	8	Error	8
Total	11	Total	11

Means will be compared using Duncan's New Multiple Range Test. The mean number of microorganisms isolated from adult female vs male guts will be compared using a t-test.

PRESENTATION OF RESULTS

A final report and manuscript will be prepared upon completion of the study.

PERSONNEL ASSIGNMENT

RWU-2203 - Responsible for all phases of study except identification of microorganisms.

J. Robert Bridges, Research Entomologist

Thelma Perry, Biological Technician

LSU-A - Responsible for identifications of microorganisms.

J. E. Marler, Associate Professor

Student Assistants

LITERATURE CITED

Barras, S. J.
1970. Antagonism between *Dendroctonus frontalis* and the fungus *Ceratocystis minor*. Ann. Entomol. Soc. Amer. 63:1187-1190.

Barras, S. J.
1972. Improved White's solution for surface sterilization of pupae of *Dendroctonus frontalis*. J. Econ. Entomol. 65:1504.

Barras, S. J.
1975a. Release of fungi from mycangia of southern pine beetles observed under a scanning electron microscope. Z. angew. Entomol. 70:173-176.

Barras, S. J.
1975b. Reduction of progeny and development in the southern pine beetle following removal of symbiotic fungi. Can. Entomol. 105: 1295-1299.

Barras, S. J. and J. E. Marler.
1974. Identification of bacterial flora in the digestive tract of the southern pine beetle, *Dendroctonus frontalis* (Zimm.). Final Report, U. S. Forest Service, FS-SO-2203-1.22, 10 pp.

Barras, S. J. and T. Perry.
1972. Fungal symbionts in the prothoracic mycangium of *Dendroctonus frontalis* (Coleopt.:Scolytidae). Z. angew. Entomol. 71:95-104.

Bramble, W. C. and E. C. Holst.
1940. Fungi associated with *Dendroctonus frontalis* in killing shortleaf pines and their effect on conduction. Phytopath. 30: 881-899.

Brand, J. M., J. W. Bracke, L. N. Britton, A. J. Markovetz and S. J. Barras.
1976. Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. J. Chem. Ecol. 2:195-199.

Brand, J. M., J. Schultz, S. J. Barras, L. J. Edson, T. L. Payne and R. L. Hedden.
1977. Bark beetle pheromones: enhancement of *Dendroctonus frontalis* (Coleoptera:Scolytidae) aggregation pheromone by yeast metabolites in laboratory bioassays. J. Chem. Ecol. 3:657-666.

Browne, L. E.
1972. An emergence cage and refrigerated collector for wood-boring insects and their associates. J. Econ. Entomol. 65:1499-1501.

Buchanan, R. E. and N. E. Gibbons (ed.).
1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

Callaham, R. Z. and M. Shifrine.
1960. The yeasts associated with bark beetles. For. Sci. 6:146-154.

Marler, J. E. and S. J. Barras.
1978. Identification of bacterial flora in galleries of the southern pine beetle, *Dendroctonus frontalis* Zimmerman. Final Report, U. S. Forest Service, FS-SO-2203-1.29, 19 pp.

Moore, G. E.
1972a. Microflora from the alimentary tract of healthy southern pine beetles, *Dendroctonus frontalis* (Scolytidae) and their possible relationship to pathogenicity. J. Invert. Path. 19:72-75.

Moore, G. E.
1972b. Pathogenicity of ten strains of bacteria to larvae of the southern pine beetle. J. Invert. Path. 20:41-45.

Nelson, R. M.
1934. Effect of bluestain fungi on southern pines attacked by bark beetles. Phytopath. Z. 7:327-353.

Nelson, R. M. and J. A. Beal.
1929. Experiments with bluestain fungi in southern pines. Phytopath. 19:1101-1106.

Norris, J. R. and P. W. Robbins.
1961. Methods in Microbiology, Vol 1, Academic Press.

Oberle, A.
1966. The microflora isolated from galleries of southern pine bark beetles, *Dendroctonus frontalis* Zimm. M.S. thesis. Northwestern State College, Natchitoches, LA.

Rumbold, C. T.
1931. Two blue-staining fungi associated with bark beetle infestation of pines. J. Agric. Res. 43:847-873.

Shifrine, M. and H. J. Phaff.
1956. The association of yeasts with certain bark beetles. Mycologia 48:41-55.

Skerman, V. B. D.
1967. Identification of the genera of bacteria. Second Ed., Williams and Wilkins Co., Baltimore.

4500
FS-SO-2203-1.42
(Problem 2 RWUD-1978)

STUDY PLAN

A QUANTITATIVE STUDY OF THE MICROFLORA ASSOCIATED WITH THE SOUTHERN PINE BEETLE

(Cooperative Agreement 19-288)

Prepared:

J. Robert Bridges — 9/22/78
J. Robert Bridges
Research Entomologist
RWU 2203

James E. Marler — 9-20-78
James E. Marler
Associate Professor
LSU - Alexandria

Approved:

Peter L. Lorio, Jr. — 10-13-78
Peter L. Lorio, Jr.
Project Leader
RWU 2203

4500
FS-SO-2203-1.42
(Problem 2 RWUD-1978)

STUDY PLAN

A QUANTITATIVE STUDY OF THE MICROFLORA ASSOCIATED WITH THE SOUTHERN PINE BEETLE

by

J. Robert Bridges and James E. Marler
(Cooperative Agreement No. 19-288 with LSUA)

INTRODUCTION

Although many types of microorganisms are associated with the southern pine beetle (SPB), the importance of most of them is not known. Three species of fungi are known to have a significant effect on beetle development. Two of these fungi are carried in the SPB female mycangium, thus ensuring a constant association with the beetle (Barras and Perry 1972; Barras 1975a). In the absence of these mycangial fungi, SPB progeny development is delayed and progeny production is decreased by more than 50% (Barras 1975b). Thus, these fungi are mutualistic symbionts and probably play a role in beetle nutrition and possibly pheromone production (Brand *et al.*, 1976). Another fungus that affects beetle development is the blue-stain fungus, *Ceratocystis minor* which is consistently found in association with the SPB (Nelson and Beal 1929, Rumbold 1931, Barras 1970). This fungi is responsible for the death of the tree and apparently aids beetle development by speeding the desiccation of the tree (Bramble and Holst 1935, Nelson and Beal 1929, Nelson 1934).

The importance of the other microorganisms found with the SPB is not known. Previous studies have identified several species of bacteria from the gastrointestinal tracts of adult SPB and from their galleries (Barras and Marler 1974, Marler and Barras 1973, Oberle 1966, Moore 1972a and b). Bacteria representing 12 genera have been isolated (Marler and Barras 1978). Some of these are potential pathogens (Moore 1972b) and some are capable of fixing nitrogen. Other species could be fortuitous contaminants which are ubiquitous in the forest environment while others might have a significant effect on beetle development.

Yeasts are also associated with the SPB (Callahan and Shifrine 1960, Shifrine and Phaff 1956), but these organisms have received relatively little study. It has been suggested that yeasts could be important in pheromone production (Brand *et al.*, 1977), but they might also play a role in beetle nutrition by producing vitamins, for example.

Qualitative studies to date have identified the species of microorganisms but have provided no information on the number of these microorganisms present at any given time. Quantitative information of the microbial population would be useful for determining which of the organisms, if any, are important to beetle development. A quantitative estimate of microbial population would also be important in the study of the succession of microorganisms in the SPB galleries.

The purpose of this study is to estimate the number of bacteria, yeasts, and fungi* present with larvae and adults of SPB and in the phloem surrounding SPB larval galleries. Microorganisms will be identified to determine which are present in the greatest numbers. Isolations will be made in galleries of larvae of various ages to determine whether there is a succession of microorganisms.

MATERIALS AND METHODS

Beetles emerging from naturally infested loblolly pine trees will be collected using a collection box similar to that described by Brown (1972). These emergent beetles will be used to inoculate freshly cut loblolly pine bolts in the laboratory. The bolts will be kept in the laboratory for various lengths of time and sampled when the bolts contain one of the following stages:

1. Eggs
2. Young larvae in larval mines before larvae have begun to enlarge their larval chamber
3. Actively feeding larvae in the larval chambers
4. Mature outer bark larvae

---

*Fungi refers to hyphal fungi as distinguished from the yeasts.

From each bolt frass from the egg niche, phloem from three sampling points (see Figure) and eggs or larvae will be collected. In bolts containing eggs and small larvae, sampling points B and C (respectively) will be in advance of gallery construction. Three galleries of each stage will be sampled. In each case the larval galleries selected for culturing will be approximately the same distance from the adult entrance hole.

Frass, phloem, and larvae will be aseptically removed and homogenized in a tissue homogenizer. Larvae will be surface sterilized (Barras 1970) prior to homogenization in order to eliminate all but the gut microorganisms. Serial 10-fold dilutions of the homogenate will be made, and 0.1 ml of each dilution will be spread in duplicate on plates of isolation media. Isolation media will include both bacteriological and fungal media. The number of cultivable bacteria, yeasts, and fungal propagules per unit of wet weight of phloem or frass will be calculated from the average number of colonies present on the primary isolation plates. Isolated colonies will be picked and obtained in pure culture by three successive passages on streak plates. Five colonies each of bacteria, yeasts, and fungi will be picked from the primary dilution plate of greatest dilution and kept for identification. Thus, the isolates to be identified will be those that are present in the greatest numbers. Isolates of the microorganisms will be identified using standard microbiological techniques (Skerman 1969, Norris and Robbins 1969, Buchanan and Gibbons 1974).

The microflora of the guts of adult SPB will similarly be studied. Guts from 5 surface sterilized emergent SPB will be pooled, homogenized, and plated as described above. Both males and females will be studied, and the procedure will be replicated 3 times.

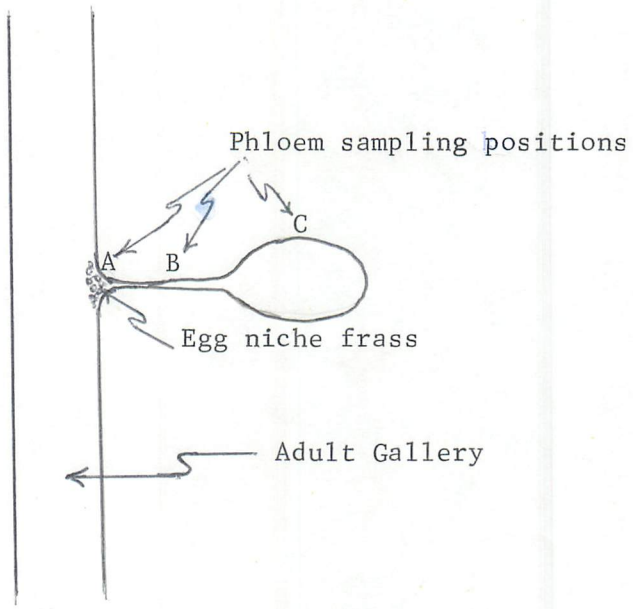

Figure. Diagram of SPB larval gallery showing sampling positions.

STATISTICAL ANALYSIS OF THE DATA

Data to be collected will include the number of cultivable bacteria (or yeasts or fungi) from each sample location. The mean number of organisms will be based on 3 replications.

	Sample positions			
Stage of bolt	Frass	A	B	C
Egg				
Larval mine				
Larval chamber				
Outer bark larvae				

For each group of organisms, these data will be analyzed using an analysis of variance with a split-plot design as summarized in the following:

Source of variation	df
Stage	3
Error I (Galleries/stage)	8
Position	3
Stage x position	9
Error II	24
Total	47

If significant interaction between stage x position is indicated, a further analysis of variance will be done as follows:

Source of variation	df
Gallery	2
Position	3
Error	6
Total	11

PRESENTATION OF RESULTS

A final report and manuscript will be prepared upon completion of the study.

PERSONNEL ASSIGNMENT

RWU-2203 - Responsible for all phases of study except identification of microorganisms.

J. Robert Bridges, Research Entomologist

Thelma Perry, Biological Technician

LSU-A - Responsible for identifications of microorganisms.

J. E. Marler, Associate Professor

Student Assistants

LITERATURE CITED

Barras, S. J.
1970. Antagonism between *Dendroctonus frontalis* and the fungus *Ceratocystis minor*. Ann. Entomol. Soc. Amer. 63:1187-1190.

Barras, S. J.
1972. Improved White's solution for surface sterilization of pupae of *Dendroctonus frontalis*. J. Econ. Entomol. 65:1504.

Barras, S. J.
1975a. Release of fungi from mycangia of southern pine beetles observed under a scanning electron microscope. Z. angew. Entomol. 70:173-176.

Barras, S. J.
1975b. Reduction of progeny and development in the southern pine beetle following removal of symbiotic fungi. Can. Entomol. 105: 1295-1299.

Barras, S. J. and J. E. Marler.
1974. Identification of bacterial flora in the digestive tract of the southern pine beetle, *Dendroctonus frontalis* (Zimm.). Final Report, U. S. Forest Service, FS-SO-2203-1.22, 10 pp.

Barras, S. J. and T. Perry.
1972. Fungal symbionts in the prothoracic mycangium of *Dendroctonus frontalis* (Coleopt.:Scolytidae). Z. angew. Entomol. 71:95-104.

Bramble, W. C. and E. C. Holst.
1940. Fungi associated with *Dendroctonus frontalis* in killing shortleaf pines and their effect on conduction. Phytopath. 30: 881-899.

Brand, J. M., J. W. Bracke, L. N. Britton, A. J. Markovetz and S. J. Barras.
1976. Bark beetle pheromones: production of verbenone by a mycangial fungus of *Dendroctonus frontalis*. J. Chem. Ecol. 2:195-199.

Brand, J. M., J. Schultz, S. J. Barras, L. J. Edson, T. L. Payne and R. L. Hedden.
1977. Bark beetle pheromones: enhancement of *Dendroctonus frontalis* (Coleoptera:Scolytidae) aggregation pheromone by yeast metabolites in laboratory bioassays. J. Chem. Ecol. 3:657-666.

Browne, L. E.
1972. An emergence cage and refrigerated collector for wood-boring insects and their associates. J. Econ. Entomol. 65:1499-1501.

Buchanan, R. E. and N. E. Gibbons (ed.).
1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

Callahan, R. Z. and M. Shifrine.
1960. The yeasts associated with bark beetles. For. Sci. 6:146-154.

Marler, J. E. and S. J. Barras.
1978. Identification of bacterial flora in galleries of the southern pine beetle, *Dendroctonus frontalis* Zimmerman. Final Report, U. S. Forest Service, FS-SO-2203-1.29, 19 pp.

Moore, G. E.
1972a. Microflora from the alimentary tract of healthy southern pine beetles, *Dendroctonus frontalis* (Scolytidae) and their possible relationship to pathogenicity. J. Invert. Path. 19:72-75.

Moore, G. E.
1972b. Pathogenicity of ten strains of bacteria to larvae of the southern pine beetle. J. Invert. Path. 20:41-45.

Nelson, R. M.
1934. Effect of bluestain fungi on southern pines attacked by bark beetles. Phytopath. Z. 7:327-353.

Nelson, R. M. and J. A. Beal.
1929. Experiments with bluestain fungi in southern pines. Phytopath. 19:1101-1106.

Norris, J. R. and P. W. Robbins.
1961. Methods in Microbiology, Vol 1, Academic Press.

Oberle, A.
1966. The microflora isolated from galleries of southern pine bark beetles, *Dendroctonus frontalis* Zimm. M.S. thesis. Northwestern State College, Natchitoches, LA.

Rumbold, C. T.
1931. Two blue-staining fungi associated with bark beetle infestation of pines. J. Agric. Res. 43:847-873.

Shifrine, M. and H. J. Phaff.
1956. The association of yeasts with certain bark beetles. Mycologia 48:41-55.

Skerman, V. B. D.
1967. Identification of the genera of bacteria. Second Ed., Williams and Wilkins Co., Baltimore.